

TECHNICAL BRIEFING:

The risks of GM crops for RNA interference (RNAi): full paper*

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The environmental and food safety of all genetically modified (GM) crops are highly questionable. GM crops with RNA interference (RNAi) constructs pose particular concerns. CSIRO's two GM wheat lines, DIR092 and DIR093 are genetically modified to use the phenomenon of RNAi to alter grain, and grain starch, composition. This type of genetic modification is complex, and the resulting GM plants highly prone to unexpected and unpredictable effects, but detailed studies of composition have not been performed. New scientific evidence indicates that the products associated with the genetically modified RNAi constructs can be taken up into the human body and may affect gene expression, raising considerable food safety concerns. However, this possibility has not been considered in the risk assessment.

The deliberate release of GM wheat containing RNAi constructs into the environment poses severe and potentially irreversible risks to the environment and human and animal health. This is especially of concern regarding the possibility of contamination of conventional wheat.

What is RNA interference?

RNA interference (RNAi) is a mechanism of gene silencing that occurs in the cells of plants and animals. In normal gene expression, the genes, made up of DNA, produce an intermediary product, "messenger" RNA or mRNA, which then goes on to be used to produce a protein. Gene silencing means that less or no protein specified by that gene is produced. This can happen by interference at the RNA level, hence the term RNAi. Although the precise mechanism for CSIRO's two genetically modified (GM) wheat lines isn't identified¹, it is generally considered that small RNAs (siRNA or miRNA), derived from double stranded RNA (dsRNA), form part of a biochemical pathway that directs them to other RNA molecules that have a sequence match². This binding prevents the mRNA from producing the protein. Although the effects of gene silencing have been observed for a considerable time, the mechanism has only been elucidated in the past decade. Small RNAs were named "Breakthrough of the Year" by Science a decade ago in 2002³. dsRNA and small RNAs are still the source of many surprises to researchers, including recent discoveries that small RNAs can be taken up by animals⁴.

How is RNA interference different from other types of genetic modification?

CSIRO's two GM wheat lines, DIR092 and DIR093⁵ use the phenomenon of RNAi to alter grain, and grain starch, composition. DNA constructs are inserted into the plant's genome. These new DNA constructs specify the production of RNA molecules that can enter the RNAi pathway. These constructs include parts of 'forward' and 'reverse' (or antisense) sequences that match parts of the target genes targeted for silencing. These forward and reverse DNA sequences each produce RNA which bind together to form dsRNA because they are complimentary to each other. The plant responds to this dsRNA by cutting it into small fragments (siRNA), and this then interferes with the expression (mRNA) of the plant's own gene which we see as gene silencing.

Genetic modification for RNAi is generally more complex than the type of genetic engineering employed by the majority of commercial GM crops that are already on the market (e.g. Roundup Ready (RR) soya or insect-resistant (*Bt*) maize). In RR soya and *Bt* maize, a gene produces a novel protein via mRNA. This protein confers a trait (e.g. in GM *Bt* maize or cotton, an insecticidal protein is produced and RR soya produces a protein that confers resistance to the herbicide Roundup). In RNAi genetic engineering, the objective is not to produce a protein, but rather to prevent a protein being produced by gene silencing.

The Office of the Gene Technology Regulator explains RNAi for the genetically modified wheat line DIR093, as follows:

Transcription of the introduced RNAi construct will generate messenger RNA (mRNA) from the tandem unit (sense) and its complimentary inverted repeat (antisense). The annealing of the sense and anti-sense mRNAs produces a double stranded RNA (dsRNA) of which the intron is spliced out. The presence

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* A summary of this paper, called "The risks of GM crops for RNA interference (RNAi): summary", is available at www.greenpeace.org/australia/en/what-we-do/Food/

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of a double stranded RNA triggers a conserved biological response to dsRNA; it is cut into small fragments of 21-25 base pairs. These small fragments guide specific enzymes to any RNA that has the same or closely similar sequence which they cleave and subsequently degrade (Ahlquist 2002). This is known as RNA interference (RNAi) and leads to the down-regulation of the targeted gene(s) (Hannon 2002). Degradation of the mRNA therefore prevents production of the targeted protein. (para 54, section 5.3)

For DIR092, RNAi is explained similarly as follows:

RNAi transgene constructs typically consist of two copies of a fragment of the target gene, arranged to give rise to a single transcript with one forward orientation copy of the target gene sequence followed by one reverse orientation copy. Because the transcript contains identical gene fragments in opposite orientations, they are complementary and naturally base-pair into a double-stranded RNA structure. The double-stranded RNA structure is recognised as being virus-like by the cellular RNAi machinery, which then cuts the transcript into fragments of 21-24 nucleotides (nt). The RNA fragments become sequence guides for enzymes which destroy complementary RNA sequences, including any endogenous transcript with sequence closely matching the transgene. Through this pathway, there is a strong decrease in levels of endogenous transcripts highly similar to the RNAi construct. Because the transcript from the construct is destroyed in this process, no proteins are produced. (para 19, section 5.1)

Concerns regarding GM plants with RNAi constructs

Whilst all GM crops are subject to concerns regarding the safety for the environment and inclusion as food for humans and animals, there are specific concerns regarding the GM wheat lines that contain RNAi constructs:

1) Effects from insertion of a complex array of genetic elements

Genetic engineering for RNAi is complex. The complexity arises because several parts of genes are required in specific senses (forward or reverse). Both DIR092 and DIR093 contain many genes and many regulatory sequences. The GM wheat DIR092 contains five partial wheat genes, several regulatory sequences and two antibiotic resistant marker genes. The GM wheat DIR093 similarly contains multiple genetic elements including regulators and antibiotic resistant marker genes.

The complexity is evident when compared with GM crops such as RR soya and *Bt* maize (see "How is RNA interference different?"). RR soya and *Bt* maize generally contain one or possibly two genes, with very few additional elements. Even in these comparatively simple GM crops, extra fragments of the inserts and re-arrangements or deletions of the plant's own DNA are known⁶. There are concerns that these irregularities may affect or interfere with the plant's metabolism, for example, by creating unintended novel mRNA⁷ or a protein, or altering an existing plant protein.

The complexity of the GM constructs required for RNAi increases the chances of unexpected and unintended effects. Disturbingly, the risk assessments for DIR092 and DIR093 appear to rely on visual evidence to detect any unintended changes.

2) Unintended changes in plant composition

Plant chemistry is complex. Altering a biochemical pathway (e.g. starch or protein production) or introducing a new biochemical pathway, has a high probability of altering another pathway. Thus, there is an issue of food safety in addition to environmental risks. For example, researchers at Monsanto tried to increase the content of carotenoids in oilseed rape (canola) but found that vitamin E decreased at the same time⁸. Another example is Golden Rice, an unexpected pathway in the rice converted lycopene (tomato red) into beta-carotene (yellow)⁹. **Therefore genetic modification of a biochemical pathway can result in unintended changes in plant composition.** Molecular profiling may help¹⁰, but criteria for evaluating the significance of any nutritional changes would have to be set.

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In the case of DIR092 and DIR093, no detailed study of composition has been conducted prior to release to the environment, only very broad analysis. Even so, changes in fat, protein and sugar levels have been observed in DIR093 (paras 84, 197 and Table 4) and changes in seed set (para 202). These nutritional changes may indicate unexpected changes at the molecular level.

3) Effects of dsRNA on non-target mRNA

The role and action of small RNAs in the functioning of genes is the subject of much research and is still poorly understood. For example, the specificity of dsRNA is unclear. One experiment¹¹ on zebrafish revealed that very small quantities could cause gene silencing and that more general changes were observed at lower concentrations than caused silencing of the target gene, suggesting specificity at these concentrations was reduced. This indicates that the GM RNAi constructs could silence other genes in addition to the target gene, known as off-target effects. This effect is acknowledged by the applicants in DIR093 (paras. 195 and 196), but no comprehensive study has been performed. Additionally, off target effects could be important if the GM wheat is ingested by animals or humans.

4) Products may pass to humans

It is claimed that no novel protein may be produced by these GM crops. However, some novel proteins may be present at low levels, below current detection limits. Even if no novel proteins are produced, food safety is still unknown. The insertion of a complex array of genetic elements and off-target effects (as described above) in this GM wheat could give rise to unexpected and unpredictable effects, limiting the value of any testing.

Of particular concern is the recent publication¹² showing the surprising result that miRNA produced in plants is resistant to digestion in the gut of animals and can be taken up into the bloodstream of the human body. The miRNA in the study was from rice that had been cooked and eaten by humans. This builds on earlier studies of other animals who also take up small RNAs from their diets into their bodies.^{13,14} The miRNA was also found to silence genes in mice, and in human tissue culture cells, providing strong evidence for the likelihood that it could affect gene expression in the human body.

The fact that small RNAs are already in the human diet does not imply any food safety. GM with RNAi constructs may create novel small RNAs that have not previously been in our diet. In this case, it would not be possible to predict whether human genes would be affected, and certainly not which genes may be affected. This is of serious concern. Even partial silencing of genes important to normal body functions might be toxic. **The possibility that the products associated with the GM RNAi constructs may enter the human body and cause toxic effects is wholly absent from the risk assessment for GM wheat with altered grain starch composition. Yet we do know it is entirely feasible.**

Insecticidal plants that utilise this dsRNA gene silencing are at the “proof-of-concept” stage¹⁵, demonstrating that products associated with GM RNAi constructs can induce toxicity in animals. Metabolic or molecular profiling may provide detailed information on composition changes induced in the GM plant but would not give any indication of toxicity to humans, farm animals or wildlife. In theory, gene expression in the consumer would have to be measured to evaluate food safety, and it's not clear whether, or how much, variation there would be between individuals.

The toxicity sections of the risk assessments for DIR 092 and DIR093 focus on the fact that no novel proteins are created by the genetic engineering (e.g. Table 4 in DIR092 and Table 6 in DIR093). Any other mode of toxicity is not considered. **This is a failing of the risk assessment, especially in light of new scientific evidence.**

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GM for RNAi poses food safety and environmental risks

Genetic modification for RNAi poses specific environmental and food safety risks above and beyond to those normally considered for GM crops. These include the complexity of the genetic modification process and off target effects by the small RNAs produced. Importantly, new scientific evidence indicates that the products associated with the GM RNAi constructs can be taken up into the human body and may affect gene expression in ways that have not yet been investigated. The mechanisms of RNAi, and in particular the mode in which RNAi products can be toxic to consumers, are currently the subject of intense scientific investigation and speculation. Given the lack of scientific information, no risk assessment is capable of assessing the risks of CSIRO's two GM wheat lines that employ RNAi to modify grain, and grain starch composition.

The deliberate release of GM wheat containing RNAi constructs to the environment poses severe, and potentially irreversible, risks to the environment and human and animal health. This is especially of concern regarding the possibility of contamination of conventional wheat. The precautionary principle should be applied and no such GM crop should be released into the environment.

Genetic modification for RNAi poses specific environmental and food safety risks above and beyond to those normally considered for GM crops.

Notes

1. Office of the Gene Technology Regulator (OGTR) applications/licences DIR 092 (<http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/dir092>) and DIR093 (<http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/dir093>). The documents referred to are the full Risk Assessment and Risk Management Plan for each line.
2. See <http://www.nature.com/focus/rnai/animations/index.html> for an animation of the process of RNA interference.
3. Couzin, J. 2002. Small RNAs make a big splash. *Science*, 298: 2296-2297.
4. Zhang et al. 2012. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. *Cell Research* 22:107-126 and corrigendum 22:273-274.
5. Office of the Gene Technology Regulator (OGTR) applications/licences DIR 092 (<http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/dir092>) and DIR093 (<http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/dir093>). The documents referred to are the full Risk Assessment and Risk Management Plan for each line.
6. Windels, P., Taverniers, I., Depicker, A., Van Bockstaele, E. & De Loose, M. 2001. Characterisation of the Roundup Ready soybean insert. *European Food Research Technology* 213:107-111. Hernández, M., Pla, M., Esteve, T., Prat, S., Puigdomènech, P. & Ferrando, A. 2003. A specific real-time quantitative PCR detection system for event MON810 in maize YieldGard based on the 3-transgene integration. *Transgenic Research* 12: 179-189.
7. Rang, A., Linke, B & Jansen, B. 2005. Detection of RNA variants transcribed from the transgene in Roundup Ready soybean. *European Food Research Technology* 220:438-443
8. Shewmaker, C.K., Sheehy, J.A., Daley, M., Colburn, S. & Yang Ke, D. 1999. Seed-specific over-expression of phytoene synthase: increase in carotenoids and other metabolic effects. *Plant Journal*, 20: 401-412.
9. Schaub, P., Al-Babili, S., Drake, R. & Beyer, P. 2005. Why Is Golden Rice Golden (Yellow) Instead of Red? *Plant Physiology* 138: 441-450.
10. Heinemann, J.A. Kurenbach, B. & Quist, D. 2011 Molecular profiling — a tool for addressing emerging gaps in the comparative risk assessment of GMOs. *Environment International* 37: 1285-1293.
11. Semizarov, D., Frost, L., Sarthy, A., Kroeger, P., Halbert, D.N. & Fesik, S.W. 2003. Specificity of short interfering RNA determined through gene expression signatures. *Proceedings of the National Academy of Sciences* 100 6347-6352.
12. Zhang et al. 2012. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. *Cell Research* 22:107-126 and corrigendum 22:273-274.
13. Zhang et al. op. cit. and Semizarov et al. op cit.
14. For a review of the heritability of RNA and transfer of dsRNA see Heinemann, J.A. 2009. Hope not Hype: the future of agriculture guided by the International Assessment of Agricultural Knowledge, Science and Technology for Development. Third World Network, Penang, Malaysia.
15. Baum, J.A. et al. 2007. Control of coleopteran insect pests through RNA interference. *Nature Biotechnology* 25: 1322 – 1326. Mao, Y-B. et al. 2007. Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nature Biotechnology* 25: 1307 – 1313. See also Gordon, K.H.J & Waterhouse, P.M. 2007. RNAi for insect-proof plants. *Nature Biotechnology News and Views* 25: 1231-123.